*Am. J. Hum. Genet. 54:397–402, 1994*

# 1993 William Allan Award Address

Antonio Cao

**Istituto di Clinica e Biologia dell'Età Evolutiva, Università degli Studi di Cagliari, Cagliari**

First of all, I would like to express my deeply felt thanks to The American Society of Human Genetics for the William Allan Memorial Award. This is a great honor, for which I feel a profound estimation. I would like also to manifest my sincere gratitude to the award committee and especially to its chairman Dr. Tom Caskey for selecting me as corecipient of this award. The topic of my talk is prevention of thalassemia major in the Sardinian population by carrier screening, genetic counseling, and prenatal diagnosis. Before describing the strategy, the organization, and the results of such a program, I would like to briefly mention where and when the rationality and the design of this program were first conceived. The place was Vienna, on the occasion of the Fourth International Congress of Birth Defects on September 1973, where I had the opportunity to meet and talk with two investigators who would have lately had a profound influence in the design and organization of such a program. The investigators were Y. W. Kan and Mike Kaback. Y. W. at that time was defining the extent of variability of the $\alpha/\beta + \gamma$-globin chain synthesis in fetal blood obtained by blind placental aspiration, in order to set up the methodology for the antenatal diagnosis of thalassemia. At that meeting, Mike Kaback presented the first promising results on the control of Tay-Sachs disease by carrier screening and prenatal diagnosis in the American Ashkenazic Jewish population. A few months later, I moved from Perugia University to Cagliari, in the island of Sardinia, where I had been nominated director of the pediatric clinic of this university. Coming back to Sardinia, where I was born, I immediately realized the magnitude and the impact, from the public-health point of view, of the β-thalassemias (table 1). At that time, indeed, 1 newborn out of 250 was affected by thalassemia major. Considering the problem in absolute terms, at least 100 new cases of thalassemia major were born per year. This, of course, created an enormous burden to our health system. You may immediately realize the magnitude of the problem, considering that the incidence of β-thalassemia in Sardinians is one order of magnitude higher than the incidence of cystic fibrosis, the most common autosomal recessive disorder in Caucasian-origin populations. In 1974, the management of the β-thalassemias consisted in regular blood transfusions and iron chelation by intramuscular desferrioxamine B. Life expectancy with this regimen does not extend beyond the second decade. This tragic reality led me to strongly believe that the only way to control this disease would have been the prevention of the birth of affected children. With this prospect in my mind I started a long and fruitful collaborative project with Y. W. Kan, in order to define molecularly the thalassemias in the Sardinian population and to set up the methodologies for their prenatal detection. We first carried out studies on the heterozygous frequency and found a figure of 12% (Cao et al. 1978), which is in full accordance with the birthrate of thalassemia major. We have also defined, by globin chain synthesis analysis, that almost all β-thalassemias in the Sardinian population belong to the $\beta^\circ$ variety, namely, β-thalassemia with completely absent production of β-globin chains (Galanello et al. 1979). We found in Sardinians that, in addition to β-thalassemia, α-thalassemia is very common and δ-thalassemia is not exceptional. As we may see later on, the interaction of α-thalassemia or δ-thalassemia with heterozygous β-thalassemia may create some diagnostic difficulties in the detection of heterozygous β-thalassemia. In the meantime, the first prenatal diagnosis of β-thalassemia was carried out, in Y. W. Kan's laboratory, in a Sardinian woman who had been identified and counseled in Sardinia by our team. M. Furbetta, a very active and dedicated member of my group, was at that time in Y. W. Kan's laboratory to collaborate with him for this project. A total of 22 women from Sardinia had prenatal diagnosis in San Francisco (Kan et al. 1975, 1977).

Received November 18, 1993. This address was delivered October 9, 1993, in New Orleans.

Address for correspondence and reprints: Dr. Antonio Cao, Istituto di Clinica e Biologia dell'Età Evolutiva, Università degli Studi di Cagliari, Via Jenner s/n, 09121 Cagliari, Italy.

© 1994 by The American Society of Human Genetics. All rights reserved.

0002-9297/94/5403-0001$02.00

**Table 1**

**Relevant Demographic Data for the Sardinian Population (1974)**

| | Data |
|---|---|
| No. of inhabitants | 1,535,724 |
| No. of newborns per year | 29,881 |
| Birthrate of thalassemia major | 1:250 |
| Total no. of new cases of thalassemia major per year | 120 |

The results were very reliable, but the sampling procedure was associated with a high risk of fetal mortality. Later on, the procedure was set up in Sardinia.

As simple and accurate methods of carrier detection were available and the facilities for prenatal diagnosis were set up, we decided that it was the appropriate time to move from retrospective diagnosis to prospective diagnosis in couples at risk identified by carrier screening. Voluntary screening was offered to prospective parents, and primarily to couples with an ongoing pregnancy. Only one member of each couple was tested, the other being examined if his/her test disclaimed a carrier state. At the beginning of our program in the mid 70s, the largest category of people requesting screening were pregnant women, whereas in the past few years the number of singles or couples without a pregnancy in progress presenting at our genetic service has been increasing continuously. This indicates a marked improvement in information on thalassemia and its prevention at the population level (Cao et al. 1981, 1991). Carrier screening and counseling were carried out at several genetic units, which were set up in the main towns of the island. In addition, in the first 3 years we organized an outreach community screening in small villages. Increased awareness of the population was achieved mostly via the mass media, namely, local newspapers, radio, TV, and magazines. A more personal approach, consisting of lectures given to the general public in factories and large stores or shops, was also frequently used. Family doctors, obstetricians, pediatricians, midwives, and nurses were all trained in this new field of preventive genetics. Posters and informative booklets were made available at critical areas such as marriage registry offices, general practitioners' surgeries, obstetricians' and pediatricians' offices, as well as family planning clinics. Information leaflets provided the following: (*a*) to whom testing will be available; (*b*) where and how to get testing; (*c*) heterozygotes are at no disadvantage; (*d*) description of the homozygous state; and (*e*) the homozygous state can be prevented safely for the mother. In addition, consultation with the parents' association was periodically organized in order to prepare the appropriate message to be given for the population education. Education on the inherited anemias, in the secondary school, by using educational videotapes, was lately introduced. An informed consent by the screenee was not requested, but, prior to testing, an effort was made to inform each person about the nature of the illness, the meaning of "carrier," the implication of being a carrier, and the alternative available to individuals found to be carriers. Critical evaluation of our sensitization program showed that the most widely used channels of information were the mass media, informing 44% of the population, followed by general practitioners (31%), obstetricians (23%), and midwives (2%). Genetic counseling was carried out in a nondirective manner to both members of the couple at risk, according to well-established principles, namely, accurate diagnosis, truth, and confidentiality. In the counseling sessions, the reproductive choices were discussed in detail, namely, birth control, adoption, artificial insemination, mate selection, and fetal testing. Full information was given regarding the risk of fetal mortality, the possible failure to obtain sufficient material for diagnosis, the risk of misdiagnosis, and the mortality and morbidity rate for any anticipated therapeutic abortion. Once identified, each carrier was informed on the implication of his/her carrier status for close relatives, and simple, clearly written material was provided for them. Relatives were informed in this way and were given the option to contact the center if they desired further information or wanted to be screened. This strategy, as we will see later on, multiplied the efficacy of the screening.

In the late 70s and early 80s fetal diagnosis was based on the analysis of fetal blood obtained by placental aspiration or fetoscopy. In this early phase of our program, the experience and skills of two obstetricians, Carlo Valenti and Giuliano Angioni, have been instrumental for our success. Fetal blood sampling is carried out only at advanced gestation (18 wk) and is associated with a high risk of fetal mortality (6%). Mostly because of fear of complications, a consistent proportion of couples counseled (7.4%) decided against prenatal diagnosis and instead continued the pregnancy. In the late 80s, when first-trimester prenatal diagnosis by chorionic villus sampling was introduced, almost all couples counseled were in favor of prenatal testing. To date, we have screened only ~13% of the Sardinian population at childbearing age. Nevertheless, we have been able to

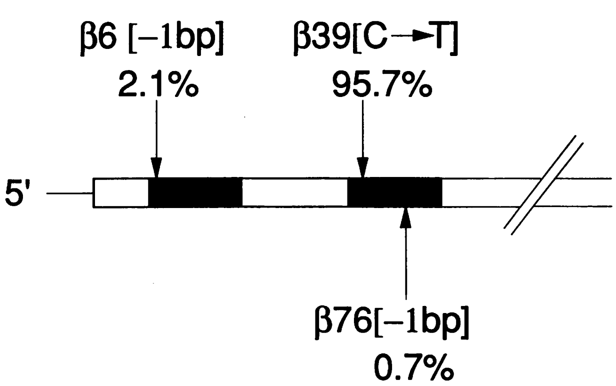

**Figure 1** Most common β-thalassemia mutations in Sardinian population.

detect 2,310 of the 2,700 couples at risk (~80%) expected on the basis of the carrier rate. The high efficiency of the screening program is related to the fact that we have largely used extended family testing (inductive screening) whenever a β-thalassemia carrier has been identified, as I have alluded to before. In the late 70s, Y. W. Kan and his coworkers detected the first polymorphic site (*Hpa*I) within the β-globin gene (Kan et al. 1978), which was found to be in linkage disequilibrium with the S mutation in the American black population. Later on, a large number of polymorphisms in this DNA region were defined. We have used polymorphism analysis to make prenatal diagnosis of β-thalassemia, for a very short period of time. The most useful site was the *Bam*HI site, which in Sardinians is in linkage disequilibrium with β-thalassemia (Kan et al. 1980).

Molecular characterization of β-thalassemia in Sardinia began in the late 70s (Trecartin et al. 1981), when Y. W. Kan cloned and sequenced the β-globin gene from a patient affected by thalassemia major. A C→T transversion was identified at codon 39, which substitutes the glutamate codon (CAG) for an amber termination codon (TAG) (codon 39 nonsense mutation). More recently, by using allelic-specific oligonucleotide probes complementary to the most common β-thalassemia mutations in the Mediterranean population, we found that the codon 39 nonsense mutation is the most frequent β-thalassemia mutation in the Sardinian population, accounting for 95.7% of the β-thalassemia chromosomes (Rosatelli et al. 1992). The second most common defect is frameshift at codon 6, which has been detected in 2.1% of the β-thalassemia genes (fig. 1). Based on these studies, nowadays, when a couple at risk is identified, we define the β-thalassemia mutation in both parents by reverse oligonucleotide hybridization using oligonucleotides complementary to the most common mutations. In those rare cases in which the mutation is not defined by this approach, we carry out denaturing-gradient-gel electrophoresis followed by direct sequencing (Rosatelli et al. 1992).

From the technical point of view, in carrier screening we encountered several technical problems, mostly related to the interaction with heterozygous β-thalassemia of α- and δ-thalassemia (Pirastu et al. 1982; Melis et al. 1983). These difficulties were overcome by investigations carried out by a team directed by one of the most brilliant of my collaborators, Renzo Galanello, to whom I am profoundly indebted for his continuous dedication to this work. The first problem derives from the relatively high incidence of the double heterozygosity for α- and β-thalassemia, which may lead to complete normalization of the red-blood-cell indices. These carriers may thus be missed by carrier screening based on MCV-MCH determination. In order not to overlook these double heterozygotes, we included HbA2 quantitation as an initial screening measure, in addition to MCV-MCH evaluation. The second technical problem we encountered was the identification of the β-thalassemia carrier state in individuals doubly heterozygous for δ- and β-thalassemia, who may have normal HbA2 levels and may thus be confused with heterozygous α-thalassemia (Pirastu et al. 1983; Galanello et al. 1988). The presence of such double heterozygotes may be suspected on the basis of family studies, which may demonstrate segregation of β- and δ-thalassemia, the latter being characterized by very low HbA2 levels. Globin chain synthesis analysis may be used to identify the presence of β-thalassemia definitively. In order to improve the ability to define δ-thalassemia, in my laboratory two young and brilliant investigators, Mario Pirastu and Paolo Moi, have recently cloned the δ-globin gene from a number of δ- and β-thalassemia double heterozygotes. It was found that, in the great majority of the cases, a single nucleotide substitution (G→T) at codon 27 of the δ-globin gene had occurred. More rarely, a 7.2-kb deletion of the ψβδ-globin region or the nonsense mutation at codon 37 were detected (Moi et al. 1988; Galanello et al. 1990; Gasperini et al., in press). Another relatively common mutation in Sardinians, characterized by normal to low HbA2 levels, is the Sardinian δβ-thalassemia. This determinant results from the presence of two mutations in the same chromosome, one of which is the β-globin codon 39 nonsense mutation and the other a C→T substitution at position −196 relative to the Cap site of the Aγ globin gene. The continuous production of Aγ chains, in adult life, from this mutated Aγ gene partially compensates the absence of β-chain production, thus explaining the mild clinical picture associated with Sardinian δβ-thalassemia (Pirastu et al. 1984*a*). Following these studies, nowadays, in a couple in which one member is a typical β-thalassemia carrier and the other is a thalassemia-like carrier with normal HbA2 and HbF, we carry out, in addition to β-globin gene analysis, also α- and δ-globin gene analysis in order to discriminate between heterozygous α-thalassemia, double heterozygosity for δ- and β-thalassemia, and γδβ-thalassemia. α-Globin gene analysis is performed on an amplified α-globin gene fragment. The presence of deletion $\alpha^{\circ}$- or $\alpha^{+}$-thalassemia is detected by PCR, using primers flanking the α-globin genes, which results in the production of unique amplified products only in the presence of the specific deletion. A DNA fragment from the normal chromosome is simultaneously amplified by using one of the primers flanking the breakpoint and a primer homologous to a DNA region deleted by the mutation. In addition, selective amplifications of α2- and α1-globin DNA, in combination with digestion by *Nco*I and *Hph*I restriction enzymes, may allow the identification of the nondeletion defects so far detected in Sardinians (Pirastu et al. 1984*b;* Paglietti et al. 1986). The presence of δ-thalassemia is defined by using allele-specific oligonucleotides complementary to the δ-thalassemia mutations defined in our population to probe an amplified δ-globin fragment.

In a proportion of the cases, homozygous β-thalassemia results in a mild clinical phenotype not requiring transfusions, which is referred to as "thalassemia intermedia." Molecular mechanisms responsible for these mild forms are inheritance of a mild β-thalassemia mutation (namely, a mutation associated with a high residual output of β-globin chains from the affected locus in the homozygous state or in the compound heterozygous state with a typical severe β-thalassemia), coinheritance with homozygosity for β-thalassemia of α-thalassemia, or other genetic determinants able to sustain a continuous production of γ chains in the adult life and, thereby, reducing the globin-chain imbalance typical of homozygous β-thalassemia. In the last few years, we have carried out studies to define the molecular mechanism for thalassemia intermedia in our population, in order to be able to improve the genetic counseling by giving a more accurate prediction of the phenotype. An ameliorating factor is certainly the coinheritance of α-thalassemia in the form of the deletion of two α-globin structural genes or point mutations in the major α2-globin gene (Galanello et al. 1989). The coinheritance of α-thalassemia, however, is not sufficiently consistent to be used to predict the phenotype. Other factors which may be useful for this purpose are the presence of frameshift at codon 6, which is in linkage disequilibrium with the C→T substitution at position −158 5′ to the Gγ gene, a mutation responsible for increased γ-chain production in conditions of erythropoietic stress, and Sardinian δβ-thalassemia, to which I have already alluded above.

**Table 2**

**Overall Results of Prenatal Diagnosis of β-Thalassemia in Sardinia**

| | FETAL DNA | | |
|---|---|---|---|
| | PCR-based Analysis | Enzymatically Restricted DNA with ASO Probes | FETAL BLOOD |
| Pregnancies monitored | 1,666 | 1,171 | 1,131 |
| Homozygous fetuses | 416 | 321 | 268 |
| Failures | | 7 (.6%) | 10 (.9%) |
| Misdiagnosis | | 1 (.09%) | 2 (.2%) |

The procedure in use for prenatal diagnosis has evolved over time. In the late 70s and early 80s, prenatal testing was carried out by globin chain synthesis analysis using fetal blood obtained by placental aspiration or fetoscopy. As soon as the molecular bases of β-thalassemia in the Sardinian population had been defined, the β-thalassemia mutations were directly detected by hybridization of allele-specific oligonucleotides to electrophoretically separated restricted nonamplified DNA fragments, according to a procedure developed by M. Pirastu in Y. W. Kan's laboratory. In the past few years, we have used $^{32}$P or horseradish peroxidase–labeled allele-specific oligonucleotide to probe fetal β-globin gene sequence of amplified DNA. The fetal DNA is obtained by transabdominal chorionic villus biopsy. Globin chain synthesis analysis has been shown to be a very reliable procedure, with only two misdiagnoses (0.2%) out of 1,131 cases monitored. The procedure is associated, however, with a very high risk of fetal mortality (6%) (table 2). Prenatal diagnosis by DNA analysis gave very reliable results. Misdiagnosis may occur for several reasons, e.g., failure to amplify the target DNA segment, mispaternity, maternal contamination, and sample exchange. In our unit, we observed only one case of misdiagnosis, resulting from maternal contami-

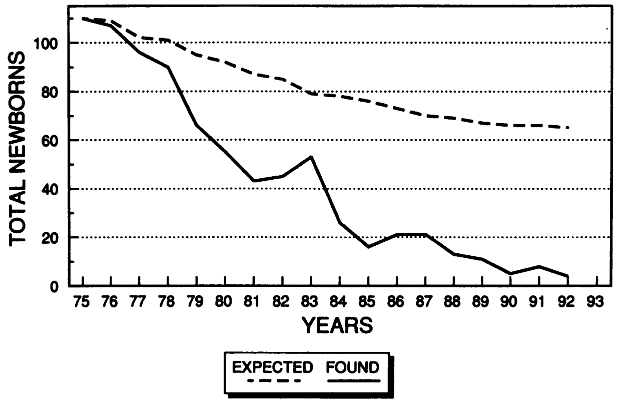

**Figure 2** Fall in the birthrate of babies with homozygous β-thalassemia in Sardinia. Absolute number of children affected with thalassemia major is plotted on the Y-axis. The carrier screening program began in 1975.

nation at the time when we were using oligonucleotide hybridization on nonamplified DNA. In order to limit the incidence of diagnostic errors, we have adopted the following guidelines: careful dissection, under the inverted microscope, of maternal decidua from fetal trophoblast; request of a minimal amount of chorionic villi (~3 μg) to limit the effect of maternal contamination; use of a limited number of amplification cycles, which may reduce the chances to coamplify DNA from maternal decidua; analysis in duplicate by using two different but overlapping amplified DNA fragments; amplification of a previously selected suitable polymorphism to monitor for the presence of maternal contamination. Overall, in the 3,968 prenatal tests we have carried out so far, 1,023 fetuses homozygous for β-thalassemia have been detected, which would have come to term in the absence of prevention. Following prenatal diagnosis of an affected fetus, the large majority (99.9%) of the couples decided to interrupt the pregnancy. Those few (0.1%) who opted for continuation of the pregnancy took this decision for ethical reasons, because of strict adherence to the Catholic religion. In this part of our program have been invaluable the technical skills, the organizing capacity, the continuous dedication to her work, and the brilliant intelligence of Cristina Rosatelli, to whom I feel profoundly indebted for our success. The great technical capability and experience of the obstetrician of our team, Giovanni Monni, gave also a fundamental contribution.

In order to monitor the efficacy of our program, we have set up a registry of new cases of β-thalassemia major in the Sardinian population. As shown in the figure (fig. 2), before prevention 1 newborn in every 250 was affected by thalassemia major. On the basis of the carriers' frequency and considering the present total number of newborns, the expected number of homozygous newborns per year should be 65. Nowadays, only 4–5 cases arise per year (1:4,000 live births), with 94% of the cases effectively prevented (fig. 2). The main reason for residual cases of thalassemia major in the Sardinian population is a complete lack of parental knowledge about thalassemia and the procedure for its prevention, followed by refusal of prenatal diagnosis, refusal of abortion when an affected fetus is identified, and false paternity (fig. 3). The results we have obtained with this 20-year program for the prevention of β-thalassemia in Sardinia indicate that programs based on carrier screening, genetic counseling, and prenatal diagnosis are very effective to control an autosomal recessive disorder, such as β-thalassemia, at the population level. Indeed, very similar results have been obtained in other Catholic, as well as Orthodox Christian and Muslim, populations composed of Greek Cypriots, Turkish Cypriots, Greeks, and Continental Italians.

The control of β-thalassemia by carrier screening and prenatal diagnosis in Sardinia may represent a model for the organization and delivery of preventive programs for other common autosomal recessive disorders, primarily cystic fibrosis. On the basis of our experience, we strongly believe that such a preventive program should be initiated when at least 90% of the molecular defects have been defined in the target population. We also believe that these programs should be preceded by an extensive educational campaign of the population at large, in order to inform them of the state of the art in this field of preventive genetics. A final critical prerequisite for a success is the education of doctors, especially obstetricians, about the technical, psychological, and social aspects of this kind of preventive medicine. However, the most important challenge for the future will

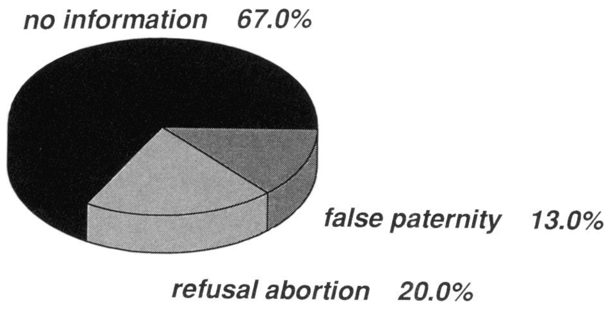

**Figure 3** Reasons for residual cases

be the organization of such a preventive program in those populations of the developing world where β-thalassemia is prevalent but where the local resources, at the present time, preclude the development of such a program.

## References

Cao A, Furbetta M, Galanello R, Melis MA, Angius A, Ximenes A, Rosatelli C, et al (1981) Prevention of homozygous β-thalassemia by carrier screening and prenatal diagnosis in Sardinia. Am J Hum Genet 33:592–605

Cao A, Galanello R, Furbetta M, Muroni PP, Garbato L, Rosatelli C, Scalas MT, et al (1978) Thalassaemia types and their incidence in Sardinia. J Med Genet 14:443–447

Cao A, Rosatelli MC, Galanello R (1991) Population-based genetic screening. Curr Opin Genet Dev 1:48–53

Galanello R, Dessì E, Melis MA, Addis M, Sanna MA, Rosatelli C, Argiolu F, et al (1989) Molecular analysis of β°-thalassemia intermedia in Sardinia. Blood 74:823–827

Galanello R, Melis MA, Podda A, Monne M, Perseu L, Loudianos G, Cao A, et al (1990) Deletion δ-thalassemia: the 7.2 kb deletion of Corfu δβ-thalassemia in a non-β-thalassemia chromosome. Blood 75:1747–1748

Galanello R, Melis MA, Ruggeri R, Addis M, Scalas MT, Maccioni L, Furbetta M, et al (1979) β°-Thalassemia trait in Sardinia. Hemoglobin 3:33–46

Galanello R, Paglietti E, Addis M, Melis MA, Tuveri T, Furbetta M, Cao A (1988) Pitfalls in genetic counselling for β-thalassemia: an individual with 4 different thalassemia mutations. Clin Genet 33:151–155

Gasperini D, Perseu L, Cossu P, Podda R, Cao A, Galanello R. A novel δ°-thalassemia mutation: TGG→TAG (TRP→STOP) at codon 37. Hum Mutat (in press)

Kan YW, Dozy AM (1978) Polymorphism of DNA sequence adjacent to human β-globin structural gene: relationship to sickle mutation. Proc Natl Acad Sci USA 75:5631–5635

Kan YW, Golbus MS, Trecartin RF, Furbetta M, Cao A (1975) Prenatal diagnosis of homozygous β-thalassemia. Lancet 2:790–792

Kan YW, Golbus MS, Trecartin RF, Filly RA, Valenti C, Furbetta M, Cao A (1977) Prenatal diagnosis of β-thalassemia and sickle-cell anemia: experience with 24 cases. Lancet 1:269–271

Kan YW, Lee KY, Furbetta M, Angius A, Cao A (1980) Polymorphism of DNA sequence in the β-globin gene region: application to prenatal diagnosis of β° thalassemia in Sardinia. N Engl J Med 302:185–188

Melis MA, Pirastu M, Galanello R, Furbetta M, Tuveri T, Cao A (1983) Phenotypic effect of heterozygous α- and β-thalassemia interaction. Blood 62:226–229

Moi P, Paglietti E, Sanna A, Brancati C, Tagarelli A, Galanello R, Cao A, et al (1988) Delineation of the molecular basis of δ and normal HbA2 β-thalassemia. Blood 72:530–533

Paglietti E, Galanello R, Moi P, Pirastu M, Cao A (1986) Molecular pathology of haemoglobin H disease in Sardinians. Br J Haematol 63:485–496

Pirastu M, Galanello R, Melis MA, Brancati C, Tagarelli A, Cao A, Kan YW (1983) $\delta^+$ Thalassemia in Sardinia. Blood 62:341–345

Pirastu M, Kan YW, Galanello R, Cao A (1984*a*) Multiple mutations produce δβ°-thalassemia in Sardinia. Science 223:929–930

Pirastu M, Lee KY, Dozy AM, Kan YW, Stamatoyannopoulos G, Hadjiminas MG, Zachariades Z, et al (1982) Alpha-thalassemia in two Mediterranean populations. Blood 60:509–512

Pirastu M, Saglio G, Chang JC, Cao A, Kan YW (1984*b*) Initiation codon mutation as a cause of α thalassemia. J Biol Chem 259:12315–12317

Rosatelli MC, Dozy A, Faà V, Meloni A, Sardu R, Saba L, Kan YW, et al (1992) Molecular characterization of β-thalassemia in the Sardinian population. Am J Hum Genet 50:422–426

Trecartin RF, Liebhaber SA, Chang JC, Lee KY, Kan YW, Furbetta M, Angius A, et al (1981) β° Thalassemia in Sardinia is caused by a nonsense mutation. J Clin Invest 68:1012–1017